WO 99/57535
PCT/US99/09793

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:
RIGAUT, Kathleen, D.
Dann, Dorfman, Herrell and Skillman
Suite 720
1601 Market Street
Philadelphia, PA 19103
ÉTATS-UNIS D'AMÉRIQUE

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year)	
11 November 1999 (11.11.99)	
Applicant's or agent's file reference	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/09793	06 May 1999 (06.05.99)	07 May 1998 (07.05.98)

Applicant
RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,EP,IL,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,HR,
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   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   11 November 1999 (11.11.99) under No. WO 99/57535

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

A-1

F ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 18 January 2000 (18.01.00)	
International application No. PCT/US99/09793	**Applicant's or agent's file reference**
International filing date (day/month/year) 06 May 1999 (06.05.99)	**Priority date** (day/month/year) 07 May 1998 (07.05.98)
Applicant WHITE, Eileen et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

07 December 1999 (07.12.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Jean-Marie McAdams
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

3057699

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N A2

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(43) International Publication Date: 11 November 1999 (11.11.99)

(21) International Application Number: PCT/US99/09793

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(30) Priority Data:

60/084,664	7 May 1998 (07.05.98)	US
60/091,391	1 July 1998 (01.07.98)	US
60/092,871	15 July 1998 (15.07.98)	US
60/107,689	9 November 1998 (09.11.98)	US

(71) Applicant *(for all designated States except US)*: RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY [US/US]; Old Queens, Sommerset Street, New Brunswick, NJ 08903 (US).

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(74) Agents: RIGAUT, Kathleen, D. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: RECOMBINANT CELL LINE AND SCREENING METHOD FOR IDENTIFYING AGENTS WHICH REGULATE APOPTOSIS AND TUMOR SUPPRESSION

(57) Abstract

This invention provides recombinant cell lines and screening methods useful for identifying agents that induce apoptosis in target cells and therefore may be used to advantage in the treatment of neoplastic disorders.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09793

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B01J 23/00, 23/40; G01N 33/48

US CL :435/326.5, 320.1; 436/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/326.5, 320.1; 436/64

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MANNERVIK, M. et al. The transcriptional co-activator proteins p300 and CBP stimulate adenovirus E1A conserved region 1 transactivation independent of a direct interaction. FEBS Letters. 1997, Vol. 414, pages 111-116, especially 111.	1-3
Y	ARNANY, Z. et al. An essential role for p300/CBP in the cellular response to hypoxia. Proceedings of the National Academy of Sciences, USA. November 1996, Vol. 93, No. 23, pages 12969-12973, especially page 12969.	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 OCTOBER 1999	Date of mailing of the international search report 09 NOV 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JENNIFER HUNT JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09793

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	THOMAS, A. et al. Suppression of the p300-dependent mdm2 negative-feedback loop induces the p53 apoptotic function. Genes and Development. 01 July 1998, Vol. 12, pages 1975-1985, especially page 1975.	1-3
Y	US 5,691,179 A (KORSMEYER) 25 November 1997, whole document.	4-6
A,E	KOSOF, G. M. Btf, a Novel Death-Promoting Transcriptional Repressor That Interacts with Bcl-2 Related Proteins. Molecular and Cellular Biology. June 1999, Vol.; 19, No. 6, pages 4390-4404, especially page 4390.	4-8

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

16

Applicant's or agent's file reference RUT 98-0058	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/09793	International filing date *(day/month/year)* 06 MAY 1999	Priority date *(day/month/year)* 07 MAY 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07 DECEMBER 1999	19 JUNE 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JENNIFER NICHOLS NEE HUNT Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/09793

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-84, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of ____

[X] the claims:
pages 85-87, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of ____

[X] the drawings:
pages 1-31, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of ____

[X] the sequence listing part of the description:
pages 1-4, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of ____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language ____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages None

[X] the claims, Nos. None

[X] the drawings, sheets/~~fig~~ None

5. [X] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/09793

V. Reas ned statement under Article 35(2) with regard to novelty, inventiv step r industrial applicability; itati ns and explanations supporting such statement

1. statement

Novelty (N)	Claims	2, 4-8	YES
	Claims	1,3	NO
Inventive Step (IS)	Claims	4-8	YES
	Claims	1-3	NO
Industrial Applicability (IA)	Claims	1-8	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1 and 3 lack novelty under PCT Article 33(2) as being anticipated by Mannervik et al. (FEBS Letters 414, 1997).

Mannervik teaches a recombinant cell line for assessing therapeutic agents that regulate apoptosis comprising a first plasmid expressing a p300 responsive promoter operably linked to a first reporter gene, a second plasmid expressing a non p300 responsive promoter operably linked to a second reporter gene and a third plasmid expressing a selectable marker gene. The cells are assessed for repression of the p300 responsive promoter.

Claims 1-3 lack an inventive step under PCT Article 33(3) as being obvious over Mannervik et al. (FEBS letters 414, 1997). Claim 2 adds the limitation of the addition of wild type p300 protein. Although Mannervik does not teach this specific addition of protein, it would be an obvious variation to add additional wild type p300.

Claims 4-8 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest the combination of Bcl-2 and Btf in an apoptosis pathway prior to the priority date claimed.

Claims 1-8 meet the criteria set out in PCT Article 33(4) for industrial applicability.

---------- NEW CITATIONS ----------

NONE

Supplemental B x
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): G01N 33/48; C12N 5/06, 5/16, 15/00, 15/09, 15/63, 15/70, 15/74 and US Cl.: 435/326.5, 320.1; 436/64

I. BASIS OF REPORT:

5. (Some) amendments are considered to go beyond the disclosure as filed:
NONE

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 295–298 (1996)
ARTICLE NO. 1795

IRES Bicistronic Expression Vectors for Efficient Creation of Stable Mammalian Cell Lines

Vanessa Gurtu,* Guochen Yan,† and Guohong Zhang,*,[1]

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Received October 9, 1996

Stable transfection of mammalian cells is a widely used technique for the study of gene expression and protein purification. However, selection of cell lines expressing desired genes from a large number of candidate clones is often labor-intensive and time consuming. To improve the efficiency of stable cell line production, we have used a bicistronic mammalian expression vector, pIRES1hyg, which contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV). The IRES element permits the translation of two open reading frames from one messenger RNA: one reading frame encoding the recombinant protein of interest and the other an antibiotic resistant marker (e.g. hygromycin). We demonstrate that the use of the bicistronic vector significantly facilitates the creation of stable mammalian cell lines, because all selected antibiotic-resistant colonies express the recombinant gene of interest. Therefore, the use of the pIRES1hyg bicistronic vector for stable transfection eliminates the need to screen large numbers of colonies to find functional clones. We conclude that the IRES bicistronic vector provides a powerful tool for efficient selection of stable transformants in mammalian cells.

Standard methods used to generate stable cell lines require transfecting a host cell line with two expression cassettes, one expressing the protein of interest and the other an antibiotic resistance marker for selection. These cassettes can be introduced into the host cell either by cotransfecting two plasmids each of which contain one of the expression cassettes, or by transfecting one plasmid containing both cassettes. Typically, after transfection and selection, approximately 5-30% of the cells functionally express the recombinant protein of interest (4,5). These relatively low frequencies can be due to many factors including deletion or inactivation of the cassette expressing the gene of interest, or, in the case of cotransfection, the stable integration of only the cassette expressing the selectable marker. Therefore, further screening of selected colonies specific for expression of the gene of interest is often necessary in order to find functional clones. Additionally, the level of gene expression using these standard methods cannot be predicted; expression is generally low and, because the selective pressure is only on the cassette that expresses the antibiotic resistance marker, expression levels can decrease over time in culture.

Unlike most eukaryotic mRNA in which ribosomes scan from the 5′ end until the initiation codon is reached, ribosomes are able to begin translation at internal ribosome entry sites (IRES) in messenger RNA of the picornaviruses (2, 3), such as encephalomyocarditis virus (ECMV). These IRES elements can be removed from their viral setting and linked to unrelated genes to produce polycistronic RNAs. In this report, we demonstrate the utility of the IRES containing bicistronic expression vector, pIRES1hyg, for the rapid and efficient generation of stable mammalian cell lines.

[1] Corresponding author. Fax: (415) 354-0776.

0006-291X/96 $18.00

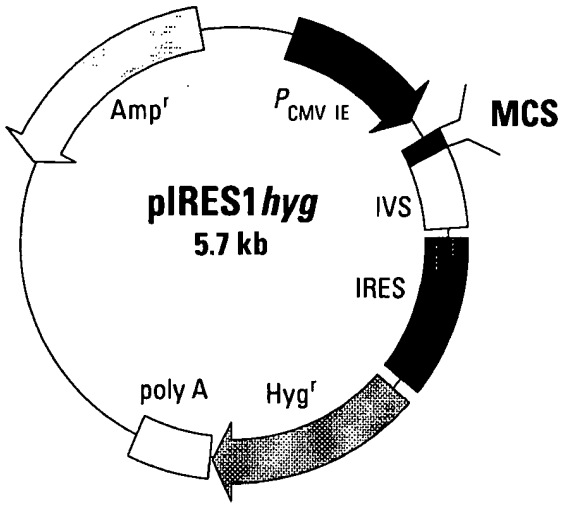

FIG. 1. Map of bicistronic expression vector, pIRES1hyg. The multiple cloning site (MCS) contains BamHI, BstXI, EcoRI and NotI sites. The internal ribosome entry site (IRES) permits the translation of two open reading frames from one messenger RNA (2, 3).

MATERIALS AND METHODS

Cell culture and reagents. CHO-K1 cells (ATCC, Rockville, MD, USA) were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin. Media, sera, and other supplements were purchased from Sigma Chemical Co. (St. Louis, MO). Cultures were maintained at 37°C with 5% CO_2/95% air.

Vector construction. All DNA manipulations were performed using standard methods (5) unless otherwise described. The pIRES1hyg expression vector was constructed by a method described previously (4). The pIRES1hyg-βgal expression vector was constructed and used to evaluate the utility of the bicistronic expression vector for the rapid and efficient production of stable mammalian cells. In the construction, the *E. coli LacZ* gene was excised by enzyme restriction in the *Not* I site of the pCMVβ expression vector (CLONTECH) and inserted into the pIRES1hyg vector through the same restriction site.

Transfection and stable selection. Transfection of pIRES1hyg was performed in CHO-K1 cells using CLONfectin transfection reagent (CLONTECH). Briefly, 8×10^5 cells were seeded in 60-mm tissue culture plates one day prior to transfection. The cultures were 60-80% confluent at the time of transfection. Cells were transfected with 6 μg plasmid DNA per plate for 2 hours. After 48 hour incubation in the appropriate growth medium, 200-600 μg/ml of hygromycin was added to the culture and selection was performed for 7-10 days.

In situ β-galactosidase staining of selected culture. Following selection, the remaining cultures were trypsinized and seeded into a new culture dish. β-galactosidase expression was detected by *in situ* staining using the X-gal substrate as described previously (6). Briefly, cells were rinsed in phosphate-buffered saline (PBS), fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS, rinsed twice with PBS, and stained for 2 hours with 0.1% X-gal in PBS containing 5 mM potassium ferricyanide and 2 mM $MgCl_2$. Cells were photographed on a Leica Leitz light Microscope (Leica Inc., Foster City, CA).

RESULTS AND DISCUSSION

To improve upon the quality and efficiency of producing stable mammalian cell lines, we have tested an IRES containing bicistronic mammalian expression vector, pIRES1hyg (Fig. 1). The pIRES1hyg expression cassette contains the human cytomegalovirus (CMV) major immediate early promoter/enhancer followed by a multiple cloning site (MCS), a synthetic intron known to enhance the stability of the mRNA (1), the EMCV IRES followed by the hygromycin gene, and the polyadenylation signal of the bovine growth hormone. The IRES element permits translation of two open reading frames: one encodes the recombinant protein of interest and the other an antibiotic resistant marker. As shown in Fig. 2, ribosomes can enter the bicistronic mRNA either at the 5′ end to translate the gene of interest or at the ECMV IRES to translate the antibiotic resistance marker.

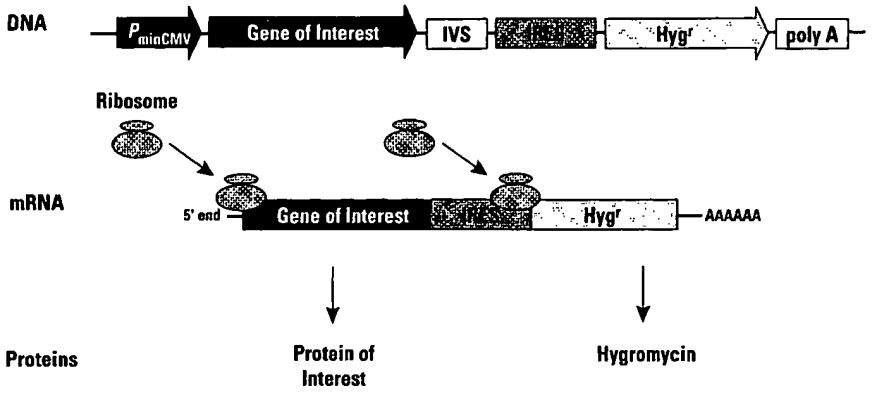

FIG. 2. Schematic diagram of the translation of a bicistronic mRNA. The open reading frames of a protein of interest and an antibiotic selection marker can be translated from the same mRNA by different ribosomes. IVS, intron; hyg, hygromycin.

To demonstrate the utility of the vector for efficient production of stable mammalian cell lines, we cloned the *E. coli LacZ* gene into the pIRES1hyg expression vector, pIRES1hyg-βgal. *In situ* detection of β-galactosidase (β-gal) expression was performed in CHO-K1 cells following transfection and hygromycin selection. We found that the percentage of β-gal positive staining cells depends on the amount of hygromycin added to the culture medium. At low concentrations of hygromycin (200-300 μg/ml), often less than 50% of the selected colonies expressed sufficient levels of β-galactosidase for detection. However, when the concentration of hygromycin was increased to 600 μg/ml, all of the surviving colonies visualized expressed sufficient levels of β-galactosidase for *in situ* detection (Fig. 3). These results demonstrate that using high doses of antibiotics selected only the cells that express sufficient high levels of β-galactosidase for detection.

Figure 4 shows a representative culture selected using 600 μg/ml of hygromycin. After 10

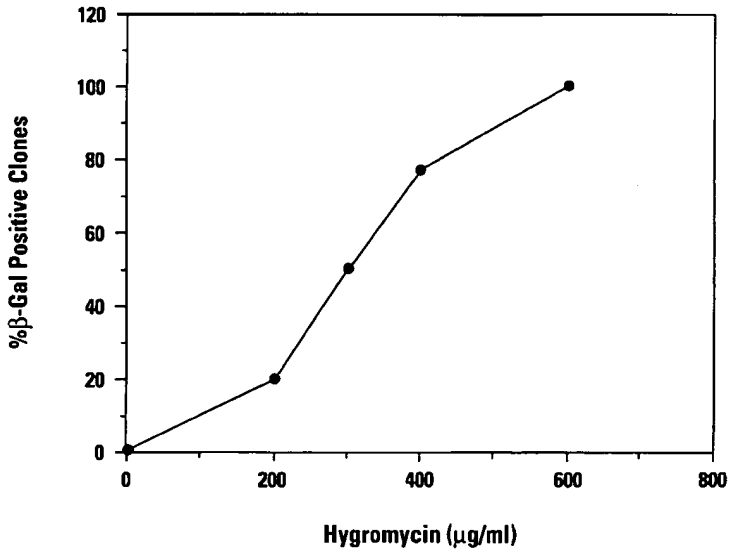

FIG. 3. Hygromycin dose-dependent selection of β-gal expressing clones. CHO-K1 cells were transfected with the pIRES1hyg-βgal expression vector as described in the Methods. Various concentrations of hygromycin were added to cultures 48 hours post-transfection and selection was performed for 7-10 days. *In situ* β-gal staining was performed as described in Methods. Generally, 4-6 representative fields for each selection were scored for β-gal expression under a Leica light microscope (Leica Inc., Foster City, CA).

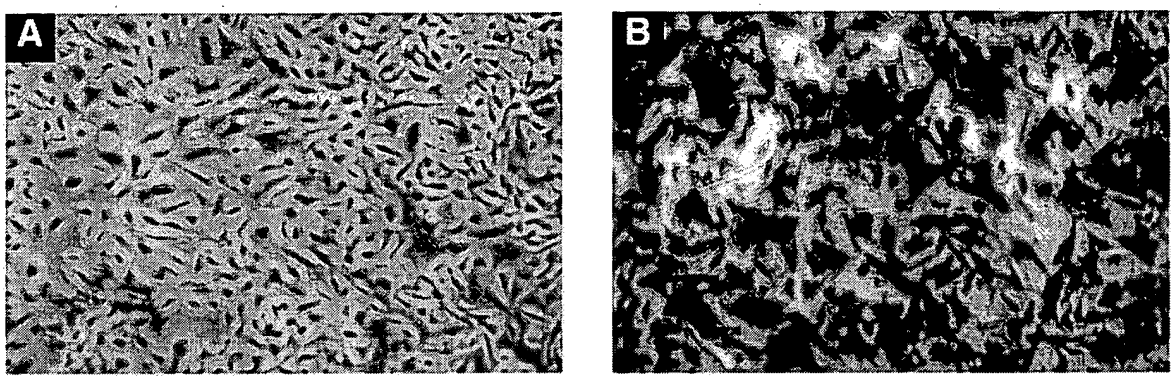

FIG. 4. Visualization of CHO-K1 cells stably transfected with the pIRES1hyg-βgal bicistronic vector. CHO-K1 cells were transfected with pIRES1hyg-βgal using CLONfectin transfection reagent as described in Methods. Antibiotic selection and *in situ* β-galactosidase staining were performed as described in Methods. β-gal staining with the selected culture shows that essentially all of the cells which survived selection express β-galactosidase (Panel B). Untransfected cultures do not stain for β-gal (Panel A).

days of selection with hygromycin, cells were trypsinized and seeded into a new culture dish. All of the surviving cells expressed β-galactosidase as shown by *in situ* β-gal staining. These results further demonstrate the effectiveness of the IRES bicistronic expression vector in the creation of functional stable cell lines. Thus, the IRES bicistronic mammalian expression vector provides an excellent system for rapidly producing stable cell lines. In addition, the IRES bicistronic expression vector has the potential to produce stable cell lines with high-level expression of the gene of interest. Since the selective pressure provided by the antibiotic is on the entire expression cassette, a high dose of antibiotic will select only for those cells expressing a high level of the gene of interest (4).

Unless a pure population of cells is required for expression experiments, the pIRES1hyg bicistronic expression vector allows researchers to use the pool of cells surviving selection, typically following 7-10 days in culture. This saves considerable time and manpower during the generation of a stable cell line, and eliminates the need for screening a large number of colonies to identify positive clones. Taken together, these studies indicate that the IRES bicistronic expression vector provides a powerful tool for the rapid and efficient production of stable mammalian cell lines.

ACKNOWLEDGMENTS

We express our appreciation to Dr. S. Rees for constructing the pIRES1hyg vector and Dr. Paul Kitts for helpful information on IRES elements. We thank Nicola Zahl, Marion Kerr and Angela Law for preparation of the figures. We gratefully acknowledge Nicola Zahl, Dr. Paul Diehl, Dr. Steven R. Kain, and Dr. John Ambroziak for critical reading of this manuscript and useful discussion.

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Cell Biology

An essential role for p300/CBP in the cellular response to hypoxia

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ABSTRACT **p300 and CBP are homologous transcription adapters targeted by the E1A oncoprotein. They participate in numerous biological processes, including cell cycle arrest, differentiation, and transcription activation. p300 and/or CBP (p300/CBP) also coactivate CREB. How they participate in these processes is not yet known. In a search for specific p300 binding proteins, we have cloned the intact cDNA for HIF-1α. This transcription factor mediates hypoxic induction of genes encoding certain glycolytic enzymes, erythropoietin (Epo), and vascular endothelial growth factor. Hypoxic conditions lead to the formation of a DNA binding complex containing both HIF-1α and p300/CBP. Hypoxia-induced transcription from the Epo promoter was specifically enhanced by ectopic p300 and inhibited by E1A binding to p300/CBP. Hypoxia-induced VEGF and Epo mRNA synthesis were similarly inhibited by E1A. Hence, p300/CBP–HIF complexes participate in the induction of hypoxia-responsive genes, including one (vascular endothelial growth factor) that plays a major role in tumor angiogenesis. Paradoxically, these data, to our knowledge for the first time, suggest that p300/CBP are active in both transformation suppression and tumor development.**

Tumor expansion beyond a certain size requires neovascularization. This process is mediated, at least in part, by local hypoxia-induced production of angiogenic factors, such as vascular endothelial growth factor (VEGF) (1, 2). Hypoxia activates the heterodimeric transcription factor hypoxia-inducible factor (HIF)-1 (3). HIF-1 is composed of α and β subunits, both of which belong to the basic helix–loop–helix (bHLH)-per-arnt-sim (PAS) protein family (3). HIF-1 binds DNA at conserved promoter/enhancer-linked HIF sites and stimulates transcription of hypoxia-responsive genes such as VEGF, erythropoietin (Epo), and various glycolytic enzymes (3). How HIF-1 activates transcription, however, is not yet understood.

Adenovirus E1A-binding p300 and CREB-binding protein (CBP) are homologous transcriptional adaptor proteins active in multiple transcriptional events (4–7). They are thought to act, at least in part, by acting as signaling conduits between specific DNA-bound transcription factors and the basal transcriptional machinery. For example, when a cell is exposed to cAMP, the heretofore inactive transcription factor, CREB, bound to a cAMP-responsive element, recruits CBP (and perhaps p300) to act as a transcriptional adaptor, and thereby stimulates transcription of cAMP-responsive genes (6, 8–10). Other factors that potentially use p300 and/or CBP (p300/CBP) as adaptors include jun, fos, c-myb, MyoD, RAR-α, SRC-1, and YY1 (11–16). In addition, p300 and CBP are both physically and functionally targeted by the adenovirus E1A and simian virus 40 large tumor (T) oncoproteins (6, 7, 17). Significantly, the ability of E1A to transform cells into a malignant phenotype requires the integrity of its p300/CBP-binding domain, implying that targeting these proteins is integral to E1A action (18). This suggests a prominent role for p300/CBP in the suppression of neoplastic transformation.

In an effort to begin to understand the p300 mechanism of action, a search for specific p300-associated proteins was initiated by an interactive protein expression cloning method (19). We used as probe a region of p300 that differs from those which serve as CREB and E1A binding sites. Using this approach, we have identified HIF-1α and subsequently found that p300/CBP and HIF-1α exist in a hypoxia-induced DNA-bound complex that appears to signal at multiple hypoxia-activated genes. These data suggest a major role for p300/CBP in the response to oxygen deprivation.

MATERIALS AND METHODS

Plasmid Constructions. Plasmids encoding glutathione *S*-transferase (GST) fusion proteins were constructed by PCR amplification of the indicated region of p300, followed by subcloning into pGEX-2TK. C/H1 (aa 300–528) encompasses the first cysteine/histidine-rich region of p300 plus approximately 100 residues on either side. C/H1Δ (contains aa 300–345 and 411–528) is equivalent to C/H1 but lacks the actual cysteine/histidine-rich region (present in residues 346–410). Recombinant baculoviruses were constructed using the BaculoGold system (PharMingen), following manufacturer's instructions; the ΔC/H1 deletion is the same as that in the GST fusion protein noted above. pCMVβ-HA-HIF-1α was created by inserting a fragment of HIF-1α cDNA into pCMVβ containing an in-frame 3′ hemagglutinin (HA) tag (4). The fragment used contains the complete ORF and the 5′ untranslated region.

Expression Cloning. GST fusion protein was synthesized in bacteria, as described elsewhere (19), using pGEX-2TK-p300C/H-1 (described above). The protein was radiolabeled *in vitro* and used to screen a 293 λZAP cDNA library (courtesy of William G. Kaelin, Dana–Farber Cancer Institute), as described (19). Excision of inserts was performed by the manufacturer's instructions (Stratagene).

Cell Culture. U-2 OS human osteosarcoma cells and Hep3B human hepatocellular carcinoma cells were maintained in DMEM/10% fetal calf serum at 37°C in 10% CO_2/90% air and 5% CO_2/95% air, respectively. sf9 insect cells were maintained in suspension at 28°C in Grace's insect medium supplemented with lactalbumin, yeastolate (GIBCO/BRL), and 10% fetal calf serum.

Electrophoretic Mobility-Shift Assay (EMSA). Nuclear extracts were prepared and EMSA assays performed as described (20), except that the final dialysis step in the nuclear extract preparation was omitted and gel conditions were as in ref. 21. The wild-type (WT) and mutant (mut) probes were

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Abbreviations: Epo, erythropoietin; VEGF, vascular endothelial growth factor; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility-shift assay.

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synthesized to match W18 and M18, respectively, in ref. 20. The XRE (xenobiotic response element) and CME (central midline element) probes were synthesized and correspond to sequences GGAGTTGCGTGAGAAGAGCCTGGAGG and AAATTTGTACGTGCCACAGA, respectively.

Monoclonal Antibodies. Monoclonal antibody, OZ15, was raised against a GST fusion protein containing HIF-1α amino acids 530–826 (Z.A. and D.M.L., unpublished results), and monoclonal antibody AC 240 was raised against a GST fusion protein containing CBP amino acids 720-1676 (R.E. and D.M.L., unpublished results).

Transient Transfections. Transfections were performed using the calcium phosphate method. Total cytomegalovirus (CMV)-bearing plasmid per transfection was kept constant at 3 μg by using pRC/CMV backbone vector (Invitrogen) as carrier. Luciferase assays were promptly performed using extract quantities normalized to the observed β-galactosidase activity (22). For the experiment in Fig. 3, cells were split into aliquots and incubated for 44 hr prior to lysis and analysis of luciferase activity. Twenty-four hours prior to lysis, one aliquot was exposed to 1% O_2, while the other remained at 21% O_2. For the experiment in Fig. 4, cells were transferred, where indicated, into 1% O_2/100 μM $CoCl_2$ 36 hr after transfection for 12–18 hr.

Northern Blot Analysis. Northern blot analysis with a VEGF probe was performed as described (23).

RESULTS

The C/H1 Region of p300 Interacts with HIF-1α. An expression library was probed with a labeled GST fusion protein encompassing the first cysteine/histidine-rich region of p300 (GST–C/H1). The longest clone (which we called CHIP-1) isolated among a set of overlapping cDNAs contained an 826-amino acid ORF predicting a protein bearing basic helix–loop–helix (bHLH) (24) and PAS domains (25). CHIP-1 cDNA was transcribed and translated *in vitro*, yielding a ≈120-kDa product (Fig. 1*A*, lane 1). This translation product was capable of binding specifically, *in vitro*, to GST–C/H1 produced in bacteria (Fig. 1*A*, lane 3) and full-length p300 produced in insect cells (Fig. 1*B*, lane 3). Binding depended on the integrity, in p300, of the first cysteine/histidine-rich region (Fig. 1 *A* and *B*, lanes 4). Similarly, CHIP-1 expressed in U-2 OS human osteosarcoma cells also bound specifically to full-length p300 produced in insect cells (Fig. 1*C*). Subsequently, the cloning of the two hypoxia-inducible factor-1 subunits (HIF-1α and HIF-1β) (3) led to the realization that CHIP-1 and HIF-1α are identical.

HIF-1α Interacts with p300/CBP During the Response to Hypoxia. Using an EMSA, we asked whether p300/CBP exists in a stable complex with hypoxia-activated HIF-1α. Nuclear extracts from both hypoxic and normoxic HeLa cells were prepared, mixed with labeled HIF probe, and electrophoresed in nondenaturing polyacrylamide gels. As shown in Fig. 2*A*, at least three specific complexes were present in normoxic extracts (lane 6, bands C, i.e., constitutive), and at least two more complexes appeared after oxygen deprivation (lane 7, bands I, i.e., inducible), as reported (28). These complexes were competed by excess unlabeled probe (lane 5) as well as probes containing cross-reacting XRE (xenobiotic response element) (29) and CME (central midline element) (26) sequences (lanes 2 and 3). The XRE sequence is bound by heterodimers of bHLH-PAS proteins (AHR and ARNT/HIF-1β) (27, 29), and the CME sequence is bound by the PAS protein product of the single minded gene (26). The complexes were not competed, however, by excess unlabeled probe containing a mutated HIF site (lane 4) or nonspecific sequences (lane 1). The fastest migrating band appears to be nonspecific, as it is competed by unlabeled excess unrelated oligonucleotide (lane 1).

To investigate for the presence of HIF-1α and p300/CBP in these complexes, we tested antibodies to these proteins as potential supershifting reagents. As shown in Fig. 2, the

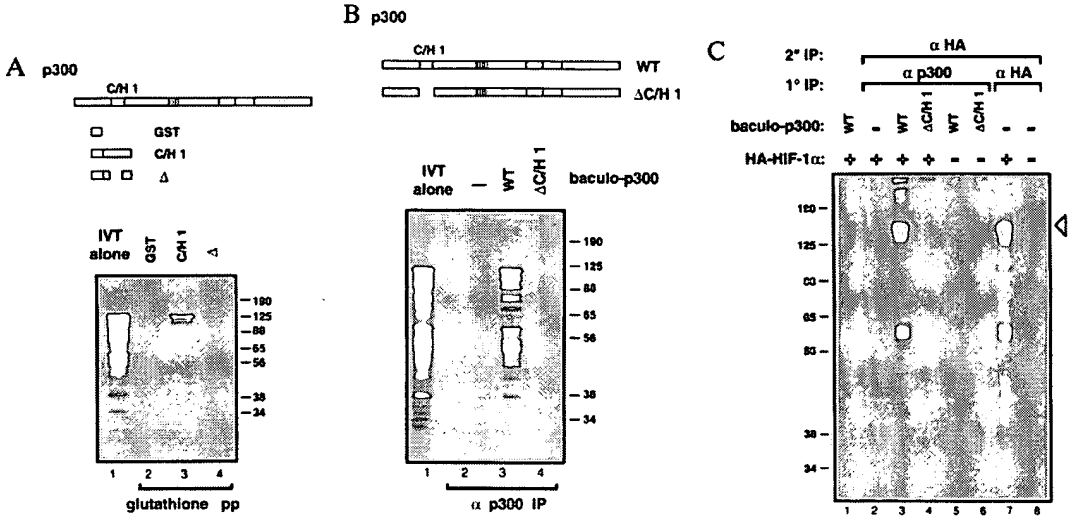

FIG. 1. p300 binds HIF-1α (28). (*A*) GST fusion proteins containing the indicated portions of p300 C/H1 were constructed. ^{35}S-Labeled HIF-1α was transcribed and translated *in vitro* and mixed with the indicated GST fusion proteins immobilized on glutathione beads. Bead-bound proteins were visualized by SDS/PAGE and autoradiography. (*B*) Full-length p300 (WT) and p300 deleted within the first cysteine/histidine-rich region (C/H1) were synthesized in insect cells using the baculovirus system. ^{35}S-labeled HIF-1α was synthesized *in vitro*, and translation products were mixed with the indicated baculo-p300 species and immunoprecipitated with a p300 antibody [RW128 (4)] using protein-A Sepharose beads. Radiolabeled bead-bound proteins were visualized by SDS/PAGE and autoradiography. Lanes 1 in *A* and *B* each contain 20% of the input translation products analyzed in the other lanes. (*C*) U-2 OS cells were transfected with 10 μg of pCMVβ-HA-HIF-1α (+) or vector alone (−). Cells were labeled with [^{35}S]methionine, and cellular extracts were prepared, mixed (where indicated) with the relevant baculovirus-p300 species, and then immunoprecipitated with either anti-p300 or anti-HA antibody, as in *B*. Bead-bound proteins were released by boiling, and reimmunoprecipitated with antibody to the hemagglutinin (HA) epitope (12CA5). Proteins bound to beads in this second round were visualized by SDS/PAGE and autoradiography. The open arrowhead indicates HA–HIF-1α. The identity of the faster migrating band is not clear but may represent a degradation product. Standard molecular weights (kDa; Sigma) are indicated. IVT, *in vitro* translate; pp, precipitation; IP, immunoprecipitation.

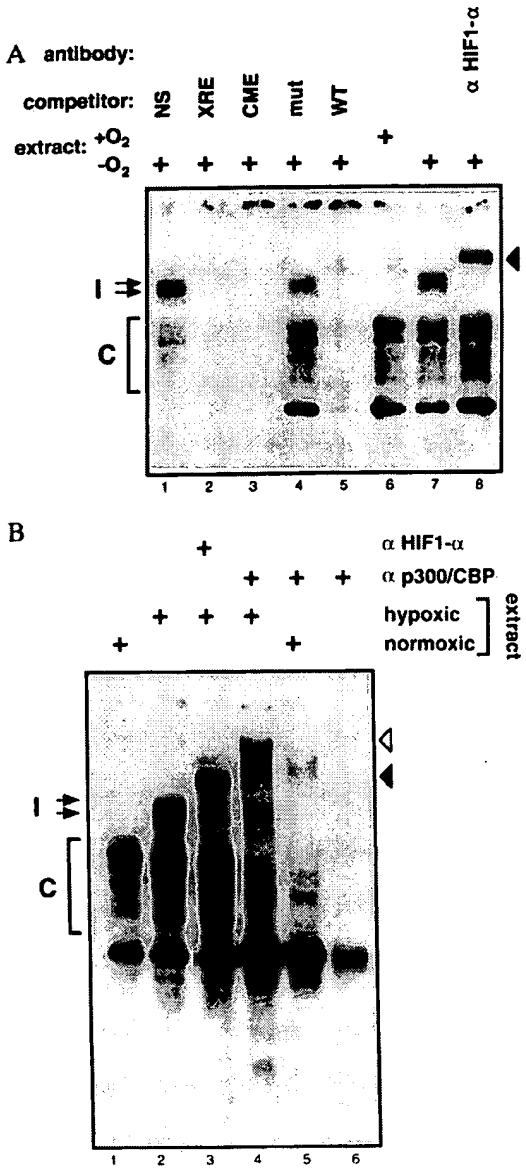

FIG. 2. One or more members of the p300/CBP family interact(s) with HIF-1α in a DNA-bound complex (27). (*A*) Nuclear extracts from Hep-3B cells were analyzed by EMSA using a HIF-site-containing probe. Extracts in lanes 1–5 and 7–8 were prepared from cells treated for 5 hr with 1% O_2, and the extract in lane 6 was prepared from normoxic cells. An 100-fold excess of unlabeled wild-type Epo 3′ enhancer probe (WT, lane 5), mutant probe (mut, lane 4), probe containing a central midline element (CME, lane 3), probe containing a xenobiotic response element (XRE, lane 2), or nonspecific probe (NS: myc E2F site, lane 1) were used as competitors. HIF-1α antibody (OZ15) was tested as a potential supershifting reagent in lane 8. In both experiments, free probe was in excess and is not shown. (*B*) Nuclear extracts from Hep-3B cells were analyzed as in *A*. Extracts in lanes 2–4 were prepared from cells treated for 5 hr with 1% O_2. Extracts in lanes 1 and 5 were prepared from normoxic cells, and lane 6 contains no cellular protein. HIF-1α antibody (OZ15) and p300/CBP monoclonal antibody (AC240) were analyzed as potential supershifting reagents in lanes 3 and 4–6, respectively. Induced (I) and constitutive (C) complexes are indicated.

hypoxia-induced complexes were supershifted by either the presence of a monoclonal antibody to HIF-1α (solid arrowheads in Fig. 2 *A*, lane 8, and *B*, lane 3) or a monoclonal antibody that reacts with both p300 and CBP (open arrowhead in Fig. 2*B*, lane 4). Hence, after hypoxia, complexes exist *in vivo* containing both HIF-1α and at least one member of the p300/CBP family.

We noted that the mobility of a fraction of the faster-migrating constitutive complexes also decreased in the presence of antibody to p300/CBP (Fig. 2*B*, lanes 4 and 5). These complexes were not supershifted by antibody to HIF-1α and, therefore, may not contain it. A similar effect was noted with another p300/CBP monoclonal antibody (data not shown). Hence, in both normoxic or hypoxic cells, p300 and/or CBP exist in complexes capable of binding to HIF sites. The detailed nature of the constitutive complexes is unclear, but one might speculate that they contain ATF-1, CREB-1, or AP-1, all of which can bind constitutively to HIF-1 sites (30, 31) and interact with CBP (and possibly p300) (6, 7).

p300 Increases the Inducibility of the Erythropoietin (Epo) Enhancer in Response to Hypoxia. Cotransfection of a reporter gene containing the 3′ Epo enhancer with pCMV-p300 resulted in a dose-dependent increase in the ratio of luciferase activity in hypoxic versus normoxic cells (Fig. 3 *Left*). Specifically, it rose from 5.3 ± 1.8 to 14.9 ± 4.8. This 3-fold increase in hypoxia induction was dependent on HIF-1, since cotransfection of p300 had no effect on luciferase production from a reporter gene in which the HIF-1 binding site was mutated (Fig. 3 *Right*). Hence, p300 increases the hypoxia inducibility of the Epo enhancer in a HIF-1-dependent fashion. The effect is most likely limited to 3-fold due to the large quantities of p300 already present in the cell, prior to transfection of pCMV-p300.

E1A Inhibits the Hypoxic Response of Both the Epo Enhancer and the VEGF Gene by Specifically Targeting p300/CBP. E1A can suppress the transcriptional activity of systems that use p300/CBP (e.g., the cAMP-mediated transcription response), as well as directly inhibit p300 transactivation function (4, 6, 7). If p300/CBP also participates in the HIF-1-mediated hypoxia response, then E1A should inhibit the hypoxia effect as well. To test this hypothesis, we transfected reporter plasmids bearing the luciferase gene under the control of the Epo enhancer linked to a thymidine kinase TATA box. As mentioned above, this enhancer contains a hypoxia-responsive HIF site. As shown in Fig. 4*A*, the activity of this reporter was stimulated 4-fold by hypoxia (bars 1 versus 2). This stimulation was completely abrogated by E1A, introduced either by coinfection (bar 3), or by cotransfection (data not shown). This inhibition by E1A was dependent on its ability to

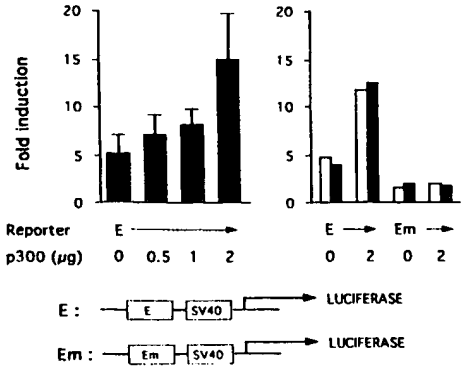

FIG. 3. Cotransfected p300 enhances hypoxic induction of transcription from the Epo enhancer (30). Transient transfection of Hep3B cells was performed with 1 μg of a luciferase reporter gene (E) containing the Epo enhancer upstream of the simian virus 40 promoter or one in which the HIF-1 binding site has been mutated (Em). Cells were cotransfected with 0–2 μg (*Left*) and 0 or 2 μg (*Right*) of pCMV-p300 (4) with 1 μg of pCMV-lacZ (to correct for variations in transfection efficiency). The *y* axis shows the ratio of luciferase expression at 1% O_2 to that at 21% O_2. Results on the *Left* are the mean of triplicate experiments ± 1 SD and on the *Right* are duplicate experimental points.

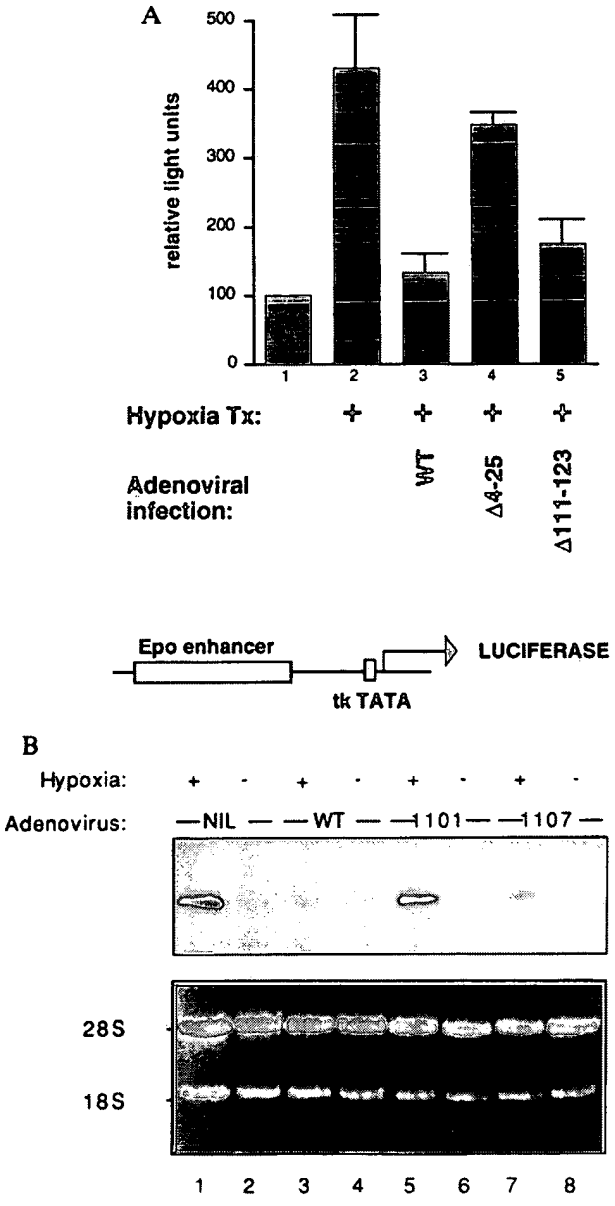

FIG. 4. E1A inhibits hypoxia-driven transcription (31). (*A*) Hep3B cells were cotransfected with 1 μg of pCMV-lacZ and 0.5 μg of Epo49-Luc (32) (diagramed below the bar graph) and brought to 16 μg of total with Bluescript DNA (Stratagene). Twenty-four hours later, cells were infected with adenovirus type 5 bearing the indicated E1A deletion mutations. The 1101 mutant deletes E1A residues 4–25, while the 1107 mutant deletes residues 111–123. E1AΔ111-123 interacts with p300/CBP but not with pRB (33), while E1AΔ4-25 does not recognize p300/CBP but does bind to pocket proteins (33). The adenoviruses also bore a mutation (dl520) rendering them unable to synthesize 13S E1A (34). (*B*) Hep3B cells were infected with either wild-type (wt) or with mutant adenoviruses as in *A*. After infection, cells were placed in either 1% or 21% O_2 for 6 hr; total cellular RNA was extracted, and Northern blots analyses using a VEGF probe were performed.

interact with p300 and CBP: adenovirus encoding a mutant E1A species selectively unable to bind p300 and CBP did not inhibit the hypoxic response (bar 4), while one encoding E1A unable to bind pRB-related proteins, but able to bind p300/CBP, did inhibit (bar 5). E1A had no effect on the reporter in normoxic cells (data not shown). We conclude that E1A abrogates the activation of the Epo enhancer in response to hypoxia and that it does so by targeting p300/CBP. In keeping with these findings, we also found that E1A was a powerful suppressor of hypoxia-stimulated endogenous Epo mRNA synthesis (L.E.H. and H.F.B., unpublished data).

Finally, we asked whether E1A also inhibits the induction of VEGF mRNA in response to hypoxia. As shown in Fig. 4*B*, infection of Hep3B cells with wild-type adenovirus markedly inhibited the rise in VEGF mRNA after hypoxia (lane 3). By contrast, a mutant virus encoding an E1A species unable to bind p300 and CBP failed to inhibit the hypoxic response (lane 5), demonstrating that the response is, at least in part, mediated by p300/CBP binding. On the other hand, an E1A species capable of interacting with p300/CBP, but defective in binding to pRB-related proteins, inhibited VEGF mRNA synthesis almost as efficiently as wild-type E1A (lane 7). Hence, as with repression of the Epo enhancer, binding of p300/CBP by E1A correlates well with its ability to repress hypoxia-induced VEGF mRNA levels.

DISCUSSION

The data presented herein suggest a critical role for p300/CBP in transcriptional regulation of hypoxia-responsive genes. p300/CBP is present *in vivo* in hypoxia-induced complexes with DNA-bound HIF-1 (Fig. 2), and it can increase the response of the Epo enhancer to hypoxia, in a HIF-1-dependent fashion (Fig. 3). Furthermore, functional p300/CBP is necessary for hypoxic induction of VEGF and Epo (Fig. 4). We conclude that p300/CBP plays a prominent role in the transcriptional response of VEGF and Epo to hypoxia and that, most likely, p300/CBP is also involved in the transcriptional induction of other hypoxia-responsive genes. The simplest model consistent with these data proposes that p300/CBP act as adaptor proteins, recruited to the HIF site by binding to HIF-1α, after which they stimulate the transcriptional machinery. In this scenario, E1A inhibits hypoxia-responsive transcription by binding to and inactivating p300/CBP. E1A most likely carries this out by directly inhibiting p300/CBP transactivation potential, rather than disturbing the p300/CBP-HIF-1α complex, since E1A can also inactivate p300 fusion proteins that probably bind to DNA constitutively (6).

As discussed earlier, p300/CBP participate in a growing variety of transcriptional regulatory systems (serum response, myogenesis, hormonal responses, etc.). Given such a wide spectrum of interactive transcription regulation function, one might speculate that p300/CBP act as scaffolds, binding simultaneously to various DNA binding (and other) transcriptional factors, and thereby integrating information from various sources. For example, p300/CBP may mediate, by binding both CREB and HIF-1α, the observed synergism during hypoxia between a cAMP-responsive element and a HIF site in the *LDHA* gene (35). In such a manner, p300/CBP may contribute to the normal integration of multiple incoming signals destined to modulate the behavior of a given promoter.

Of what advantage to the virus is the E1A blockade of hypoxia-responsive gene activation? One possibility is that the response to hypoxia is deleterious to viral replication (36, 37). Neutralization of hypoxia-induced genes might, thus, favor viral production. Alternatively, the effect of E1A may simply be an epiphenomenon of its concomitant effect on a different regulatory system which also involves p300/CBP. For example, p300/CBP are also adaptors in the interferon α (IFN-α) pathway (38), which has potent antiviral proliferative effects. Inhibition by E1A of the IFN-α pathway is well documented (39–41). Thus, inhibition by E1A of hypoxia-responsive genes may simply be secondary to its more purposeful inhibition of IFN-α-responsive genes.

The data presented herein imply strongly that p300/CBP contribute to the hypoxia-induced activation of the VEGF promoter and, hence, to VEGF synthesis. Hypoxia-induced elaboration of VEGF is believed to play a pivotal role in

hypoxia-induced angiogenesis and, hence, tumor expansion (1, 2). Thus, p300/CBP, a known transformation-suppressing element, may have the paradoxical feature of supporting tumor progression. In such a setting, the p300/CBP–HIF complex, a hypoxia-specific structure, might become a possible target for rational therapy of tumors and other disorders characterized by aberrant hypoxia-induced neovascularization.

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Binding and modulation of p53 by p300/CBP coactivators

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The adenovirus E1A and SV40 large-T-antigen oncoproteins bind to members of the p300/CBP transcriptional coactivator family. Binding of p300/CBP is implicated in the transforming mechanisms of E1A and T-antigen oncoproteins. A common region of the T antigen is critical for binding both p300/CBP and the tumour suppressor p53 (ref. 1), suggesting a link between the functions of p53 and p300. Here we report that p300/CBP binds to p53 in the absence of viral oncoproteins, and that p300 and p53 colocalize within the nucleus and coexist in a stable DNA-binding complex. Consistent with its ability to bind to p300, E1A disrupted functions mediated by p53. It reduced p53-mediated activation of the *p21* and *bax* promoters, and suppressed p53-induced cell-cycle arrest and apoptosis. We conclude that members of the p300/CBP family are transcriptional adaptors for p53, modulating its checkpoint function in the G1 phase of the cell cycle and its induction of apoptosis. Disruption of p300/p53-dependent growth control may be part of the mechanism by which E1A induces cell transformation. These results help to explain how p53 mediates growth and checkpoint control, and how members of the p300/CBP family affect progression from G1 to the S phase of the cell cycle.

When stabilized by T antigen, p53 was found to bind members of the p300/CBP family (p300, CBP and p400)[1,2]. We investigated whether non-T antigen-mediated stabilization of p53 enables p53–p300/CBP complexes to be detected. Complex formation was assessed in ts20TG^R cells[3], which are temperature sensitive in the E1 ubiquitin-activating function, and accumulate stable p53 at 39.5 °C. Endogenously labelled p400 and CBP co-immunoprecipitated with p53 (Fig. 1a, compare lanes 4 and 8 with 2; also data not shown), and p53 co-immunoprecipitated with p300/CBP family members (Fig. 1a, lanes 6 and 7) at the non-permissive temperature. Unlike CBP, p300 co-immunoprecipitated with p53 at both temperatures by p300-specific immunoblotting (Fig. 1b, lanes 4 and 6), suggesting that detection of p300/p53 binding is not dependent on p53 metabolic stabilization. Taken together, the data show that stable p53–p300/CBP family-member complexes form in the absence of T antigen. A comparison of the quantities of p53-coprecipitated p300 and total p300 available (Fig. 1b) suggests that ≤1% of total cellular p300 coprecipitated with p53 under the conditions used.

E1A is known to inhibit p53 transcription-activation function[4], and binds to a specific domain (C/H3) of p300/CBP[5]. Given that p53 also binds p300/CBP family members, we investigated whether E1A inhibits p53 transcription by binding C/H3 and downregulating p300/CBP-mediated p53 coactivation. U-2 OS cells, which synthesize wild-type p53, were transfected with a consensus p53 binding site-containing CAT reporter (PG_{13}-CAT), or its mutant counterpart (MG_{15}-CAT), which is not responsive to p53 (ref. 6). Wild-type 12S E1A specifically inhibited p53-mediated activation of PG_{13}-CAT (Fig. 2a). E1A mutant Δ2-36 failed to repress p53-dependent transcription. This mutant binds to pRB, but not to p300/CBP family members[7]. The E1A mutant, CXd1, which binds p300/CBP but not proteins of the retinoblastoma family, fully repressed promoter activity. These data indicate that one or more p300/CBP family members may coactivate p53. Mutant E1A Δ26–35 is defective in binding to p400 but not p300 (ref. 8), yet E1A Δ26–35 actively repressed p53-dependent transcription. Hence p400 is not the only p300 family member responsible for p53 coactivation.

PG_{13}-CAT and two other p53-responsive reporter plasmids, pWWP-luc, which carries the $p21^{WAF1/cip1}$ promoter[9], and pTM667-3, which carries the *bax* promoter[10], were transfected with a p53 expression plasmid into p53-null Saos-2 cells (Fig. 2b–d). Again, E1A repression of p53-mediated transcription activation correlated with its ability to bind p300/CBP family members. Hence the same genetics of E1A repression of p53 transcription activation apply to two naturally occurring p53-activated promoters. Therefore, p300/CBP family members are implicated as modulators of p53-dependent transcription-activation function.

Overproduction of wild-type p300 overrode E1A-mediated repression of PG_{13}-CAT (Fig. 2e). The mutant p300 species, del33, which lacks an intact C/H3 domain and cannot bind to E1A[5], failed to override repression. This is consistent with the view that E1A represses p53-mediated transcription activation by binding p300/CBP family member(s). The data are also consistent with a model in which the C/H3 domain of p300/CBP contributes to p53 coactivation, and E1A inactivation of this domain is relieved through sequestration of E1A by overexpressed p300. C/H3 is also the

Table 1 E1A override of p53-induced G1 cell-cycle arrest and apoptosis

		Transfected cells (%)			
Experiment code	Transfected DNA	sub-G1 (apoptotic)	G1	S	G2/M
A	pCMV	7.24	21.4	26.5	52.1
	p53	17.72	43.9	14.3	41.9
	p53 + wt E1A	7.30	28.8	17.1	54.2
	p53 + E1A Δ 2-36	14.08	41.4	19.3	39.4
B	pCMV	7.66	31.5	36.4	32.0
	p53	18.04	47.2	16.7	36.0
	p53 + wt E1A	10.80	34.5	29.3	36.2
	p53 + E1A Δ 2-36	16.10	45.3	21.5	33.3

Results are for Saos-2 cells. The G1, S and G2/M values are percentages of the non-apoptotic transfected cell population.

binding site for the histone acetylase P/CAF[11], suggesting that p53–p300/CBP transcription signalling might involve chromatin remodelling by P/CAF.

To determine whether a p300 family member is a component of a stable p53-DNA-binding complex, electrophoretic mobility shift assays (EMSA) were performed (Fig. 3a), using as the DNA probe a p53-binding site from the p21 promoter, together with nuclear extract from Saos-2 cells producing ectopic p53. A p53–DNA complex was observed (lane B, open arrow). Complex formation was p53 dependent (data not shown) and sequence specific (compare lanes C and D). The complex was specifically supershifted by the p53-specific antibodies pAb 421 (not shown) and pAb 1801 (compare lanes B and E, filled arrow). Anti-p300/CBP antibodies[12] TAP p300L and AC 238 eliminated the p53–DNA complex (lanes F and G). The p300 antibody RW 128 did not perturb the complex (lane H), possibly because of epitope masking. Importantly, AC238 specifically super-supershifted the pAb 1801-supershifted complex (compare lanes J and N, bracketed region). Taken together, these data suggest that nearly all of the DNA-binding p53 detected in this assay is complexed with a p300/CBP family member. E1A failed to disrupt any of these complexes (lanes P, Q and S). Therefore, disruption of the complex is probably not the basis for E1A inhibition of p53-mediated transactivation.

The existence of p53–p300/CBP-family-member complexes was further supported by the intracellular localization of p53 and p300 in cotransfected U-2 OS cells (Fig. 3b–d). Green fluorescent protein-tagged p53 (GFP–p53) yielded a diffuse nuclear green fluorescence (Fig. 3b, left). Cotransfection of haemagglutinin-tagged wild-type p300 brought GFP–p53 into nuclear 'dots' (Fig. 3c, left), to which p300 colocalized (Fig. 3c, middle and right). When overproduced, p300 exists in such dots[5,13]; p130 is excluded from them[13], implying that they are p300 specific. When wild-type E1A was cotransfected with GFP–p53 and epitope-tagged p300, neither p300 nor p53-containing dots were detected (Fig. 3d, right and left, respectively). In contrast, cotransfection of Δ2–36 E1A, which is unable to bind to p300, together with GFP–p53 and epitope-tapped p300, failed to disrupt either the p300 dots or their recruitment of GFP–p53 (data not shown). Similar results were obtained in Saos-2 cells (data not shown). These data reveal a strong correlation between recruitment of p53 into p300 nuclear dots and the ability of p53 specifically to transactivate certain target promoters, such as *p21* and *bax*.

Finally, we investigated whether two p53-associated biological functions, G1 arrest and induction of apoptosis, were mediated by p300. Overproduction of wild-type p53 in Saos-2 cells induces G1 arrest[14]. This effect was relieved by cotransfection of wild-type 12S E1A, but not by Δ 2–36 (Table 1). Similarly, wild-type E1A, but not Δ 2–36 E1A, suppressed p53-induced apoptosis at early times after transfection (Table 1). Therefore, the p300/CBP contribution to p53 transcription function may underlie the ability of p53 to arrest cells in G1 and to induce apoptosis. It has been suggested that p53 can induce apoptosis through two mechanisms, one of which is dependent

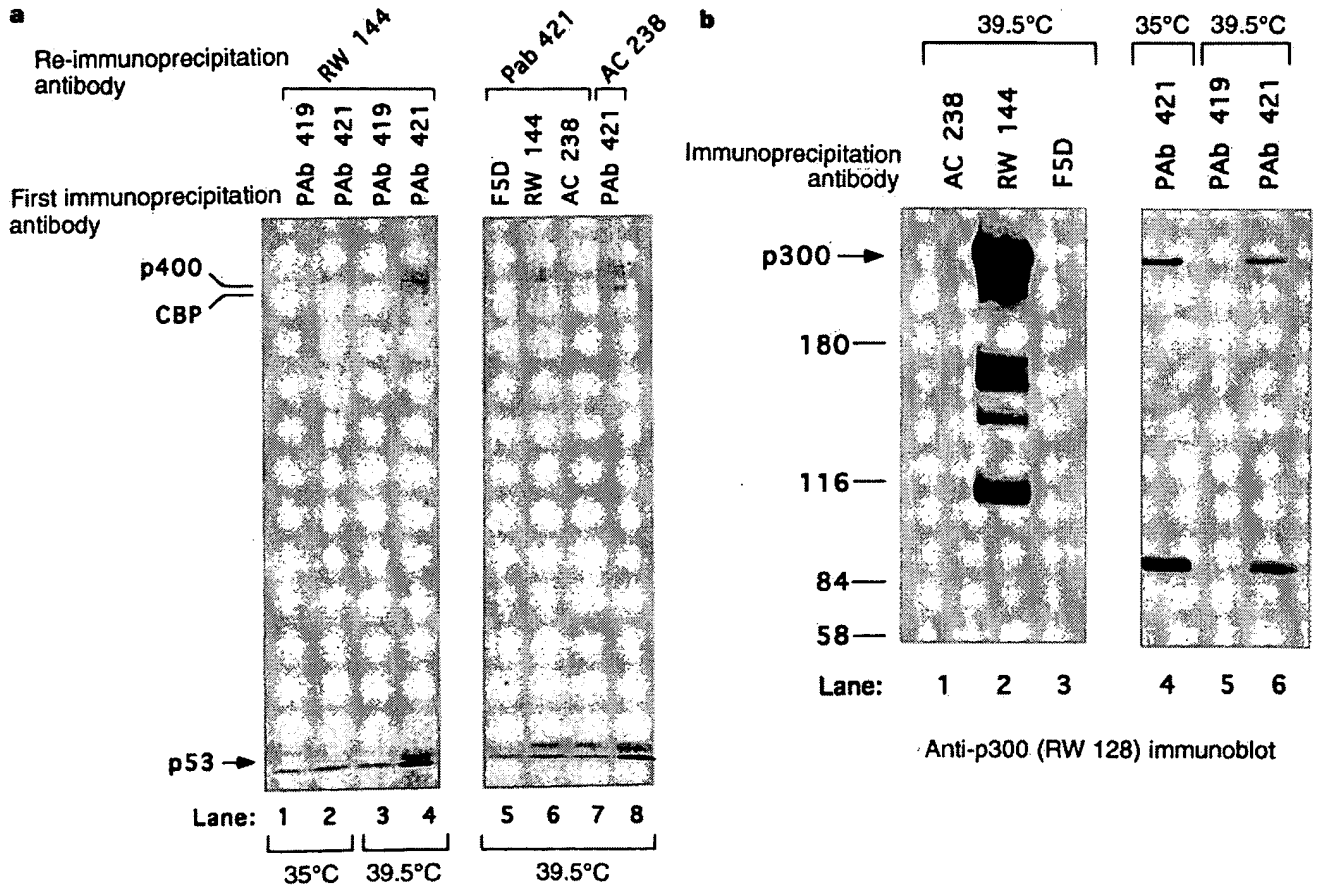

Figure 1 p53–p300 family member complexes form without viral oncoproteins. **a**, Immunoprecipitation/re-immunoprecipitation of ^{35}S-labelled proteins; ts20TG^{R} protein amounts were 2 (39.5 °C) or 3.8 mg (35 °C). Reactions using 35 °C extracts contained half the p53 present in those using 39.5 °C extracts (not shown). **b**, Immunoprecipitation/RW 128 immunoblotting. Immunoprecipitates were prepared from unlabelled ts20TG^{R} extract proteins (35 °C, 25 mg, lane 4; 39.5 °C, 15 mg, lanes 5 and 6). RW 128 immunoblotted p300 (lane 2) but not CBP (lane 1). Lanes 1–3 show immunoprecipitates from 150 μg of 39.5 °C extract protein. The protein of ~90K in lanes 4 and 6 is unidentified, and may be a p300 degradation product.

on p53 transcription activation[15,16]. The induction of early apoptosis (<48 h) by p53 is transactivation-domain dependent, whereas late apoptosis (>72 h) is not[16]. E1A-mediated suppression of apoptosis in Saos-2 cells was apparent at 48 h after transfection and was barely observable 72 h after transfection (data not shown). Therefore, p53/p300 complex formation may significantly influence transcription-dependent early apoptosis.

Our results strongly suggest that the ability of p53 to mediate important functions, such as its DNA-damage/G1-checkpoint-activation function, is p300/CBP dependent. p300/CBP function has been linked independently to the control of both G1 exit and genome stability[17,18]. These two regulatory activities probably depend on an effective checkpoint-maintenance function in which p53–p300/CBP complexes seem to be important. It will be interesting to determine whether p300/CBP directly mediate(s) p53 transcription signalling to the core transcription apparatus and more specifically, whether this involves the known interactions[19,20] of p300/CBP and p53 with TBP, TAFs or P/CAF[11].

Note added in proof: While this manuscript was under review, it was reported that E1A inhibits p53-mediated p21 transactivation and cell-cycle arrest through its p300/CBP-binding domain[30]. □

Methods

Plasmids. Reporters PG_{13}-CAT[6], MG_{15}-CAT[6] and pWWP-luc[9], and wild-type p53 expression vector pC53-SN-3 (ref. 21) were provided by B. Vogelstein. The *bax* reporter, pTM667-3 (ref. 10), was provided by J. Reed. The pCMV12S[22] and pE1A.CXdl vectors[7] were provided by E. White. pCMV12S encodes wild-type 12S E1A. The E1A CXdl mutant lacks amino acids 121–150. E1A Δ2–36 and Δ26–35 coding sequences were derived from plasmids provided by E. Moran[7] and S. Bayley[8], respectively. The pCD-GFPp53 vector encoding GFP–p53 was generated by insertion of a GFP cassette[23] (provided by P. Silver) into expression vector pCDNA3-p53 (provided by W. G. Kaelin Jr and C. Jost). The CD20 expression vector, pCMVCD20 (ref. 24), was provided by L. Zhu. pBSK has been described (Stratagene, pBluescript SK).

Cells. The ts20TG^R cells, a gift of H. Ozer, have been described[3]. Saos-2 and U-2 OS human osteosarcoma cell lines were obtained from the American Type Culture Collection.

Antibodies. The anti-p300/CBP ascites used in this study were described previously[5,12]. TAPp300L was provided by M. Ewen. AC 238 and RW 128 ascites recognize both p300 and CBP in EMSA assays[12]. For immunoprecipitations, hybridoma supernatant AC 238 (IgG1) is CBP specific, whereas RW 144 (IgG1) is p300/p400 specific. Hybridoma supernatant RW128 is p300 specific in immunoblotting. The antibodies PAb 421 (IgG2a, anti-p53) and pAb419 (control IgG2a, anti-SV40 T antigen) were provided by E. Harlow. Anti-myogenin antibody F5D (control IgG1) was provided by W. Wright. Anti-CD20 antibody was obtained from Dako (M0774). Antibody 12CA5 (BAbCo) recognizes the haemagglutinin epitope. Anti-p53 antibody pAb 1801 was obtained from Santa Cruz.

Immunoprecipitation/re-immunoprecipitation. Experiments were performed as described[1], using cells incubated at the indicated temperatures for 16 h.

Transfection. Transfections were performed using the calcium phosphate

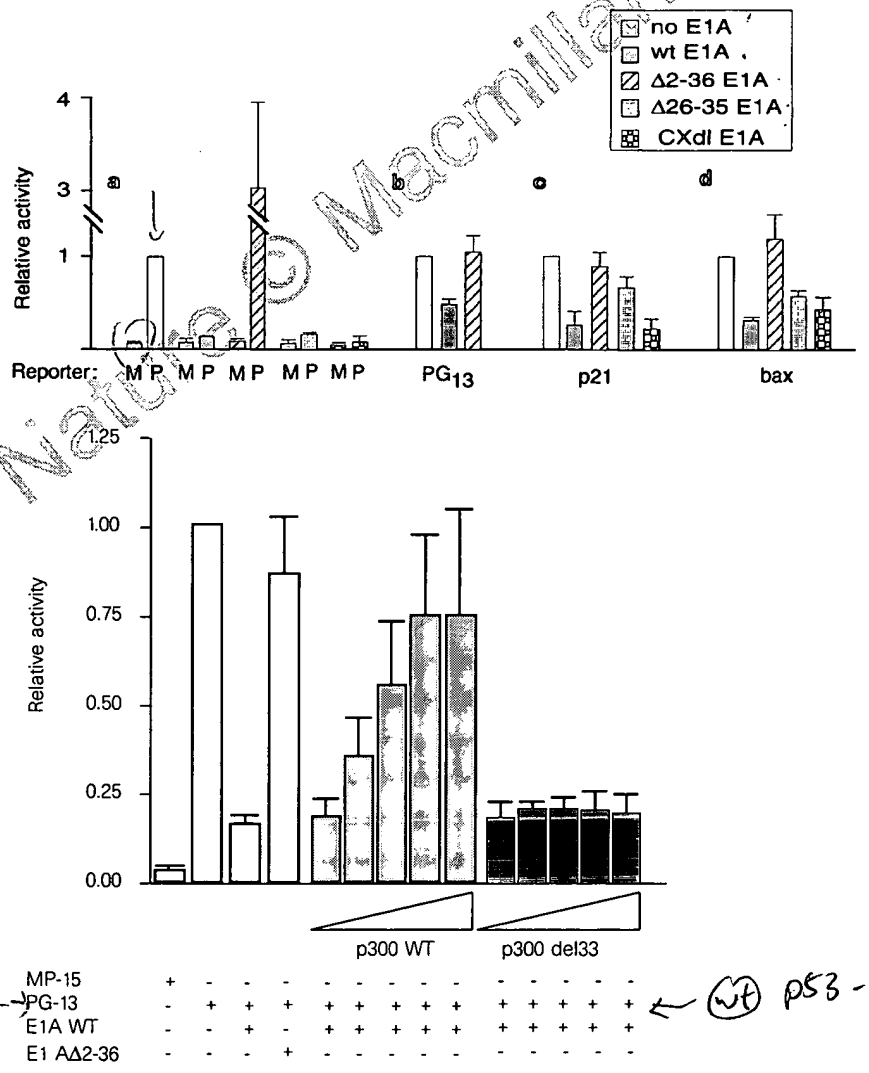

Figure 2 Genetics of E1A-mediated inhibition of p53-responsive reporters. **a**, U-2 OS cells received 2 μg of reporter (M, MG_{15}; P, PG_{13}), 0.5 μg of E1A expression vector, or equimolar pCMV vector. DNA (7.5 μg) was added to 60-mm dishes. **b**–**d**, Saos-2 cells (10-cm dishes) were transfected with 5 μg reporter, 1 μg E1A expression vector, and pC53-SN-3 (pCMV p53; 2 μg for b; 0.5 μg for **c** and **d**) or equimolar pCMV vector; 18.5 μg of DNA was added per dish. Reporter plasmids were: **b**, PG_{13}-CAT; **c**, pWWP-luc; **d**, pTM667-3. Results are averages of three experiments for **a**–**c**, and two experiments for **d**. For **b** and **d**, relative activity in the absence of p53 was less than 0.01. For **c**, the highest activity in the absence of p53 was 0.13. **e**, U-2 OS cells (10-cm dishes) were transfected with 5 μg reporter and 0.1 μg E1A expression vector; 17.1 μg DNA was added per dish. Increasing amounts (0.1, 2, 6, 10 and 12 μg) of p300 expression vector were added as shown. Ectopic proteins (wild-type p300 (WT) and p300 del33) were comparably expressed (not shown). Results are averages of three experiments, each with duplicate samples. Error bars, s.d.

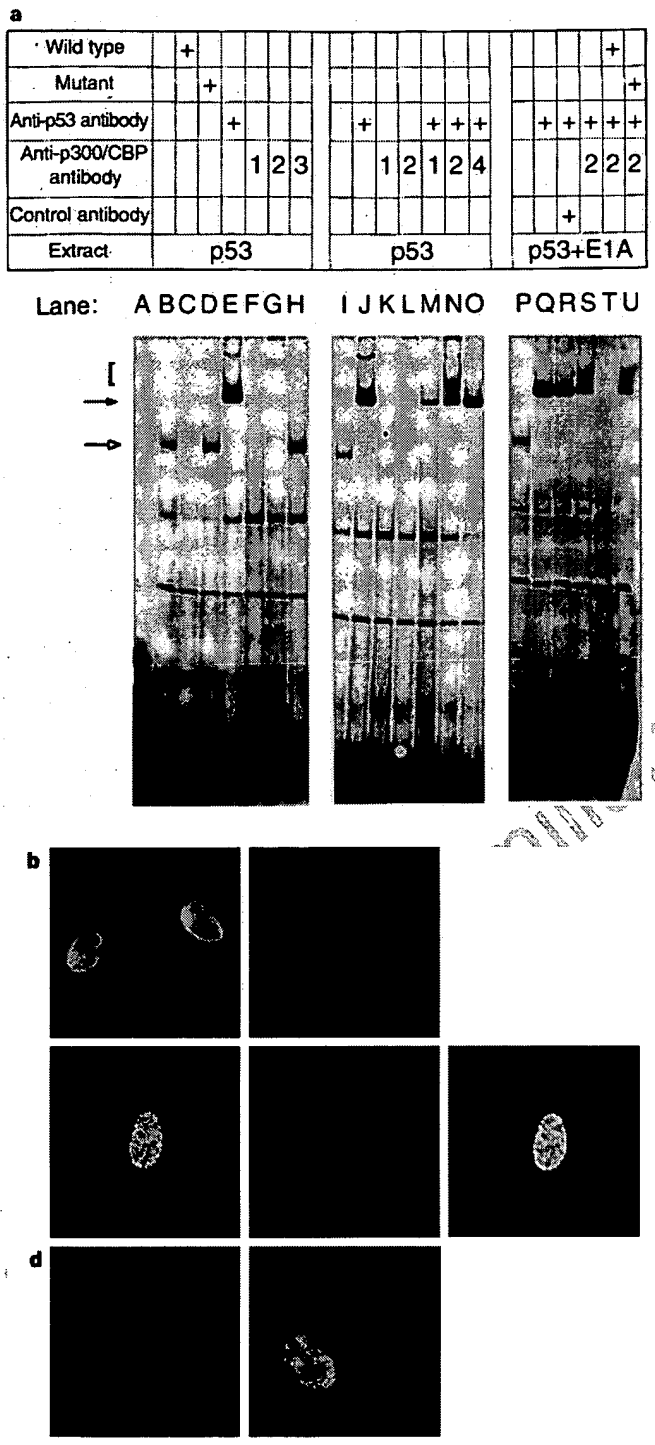

Figure 3 Colocalization of p300 and p53 in DNA-binding complexes and nuclear dots. **a**, EMSA using a p21 promoter p53-binding site. Saos-2 cells (10-cm dishes) were transfected with pC53-SN-3 (pCMV p53, 0.5 μg), or pC53-SN-3 plus pCMV12S (pCMV wild-type E1A, 1 μg). Probe alone, lane A. Anti-p300/CBP antibodies: 1, TAPp300L; 2, AC 238; 3, RW 128; 4, AC 240. Control antibody, F5D. 'Wild-type' and 'mutant' indicate competitor DNA added. Three gels are shown from a single experiment. **b–d**, Immunofluorescence of transfected U-2 OS cells. Green, GFP-p53; red, haemagglutinin-tagged p300; yellow, overlapping red and green fluorescence. Transfected DNAs: **b**, pCD-GFPp53 alone; **c**, pCD-GFPp53 and CMV-β p300-CHA; **d**, pCD-GFPp53, CMV-β p300-CHA and pCMV12S (wild-type E1A).

method. Input doses of the pCMV promoter were equalized with pCMV-β, and total DNA was equalized with pBSK.

Reporter assays. Chloramphenicol acetyltransferase (CAT) and luciferase were assayed as described[25]. Extracts were prepared 44–48 h after transfection. Equal quantities of extract protein were analysed in each assay. Results are given as relative activity, based on the positive control activity (arbitrarily set at 1) observed for each p53-responsive reporter in a given cell type in the presence of p53 and the absence of E1A.

Electrophoretic mobility shift assay. Nuclear extracts[26] were prepared from Saos-2 cells approximately 44 h after transfection. For DNA-binding reactions, 4 μg of extract was used. Procedures were similar to those described[27]. Reaction mixtures contained 18 μl of binding buffer (50 mM KCl, 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 10% glycerol, 0.5 mM dithiothreitol, 0.1% Nonidet P-40), plus 1 μg sonicated DNA from salmon testes. The double-stranded p21 oligonucleotide probe (and wild-type competitor) was 5′-AATTCTCGAG-GAACATGTCCCAACATGTTGCTCGAG-3′ (ref. 28). The mutant competitor double-stranded oligonucleotide, MG_{15}, was 5′-CCTTAATGGACTT-TAATGGCCTTAATGGACTTTAATGG-3′ (ref. 6). Competitor DNA and/or antibody was incubated with binding buffer for 15 min before nuclear extract was added. Anti-p300/CBP ascites fluids were added at 2 μl per binding reaction. The antibody, pAb 1801 was added at 2 μl (0.2 μg) per reaction. Mixtures containing extracts were incubated for 15–20 min at 23 °C before the addition of 1 ng of radiolabelled probe. Competitor DNA was present in a 100-fold excess relative to the labelled probe.

Immunofluorescence. U-2 OS cells in 6-well plates were transfected with (μg DNA per well): 0.1 pCD-GFPp53, 2.0 CMV-β p300-CHA, 0.1 pCMV12S or Δ2–36. The total DNA was 6 μg per well. Cells were fixed and immunostained ~40 h after transfection[12]. p300-CHA was detected using 12CA5 and rhodamine-conjugated secondary antibody.

Cell-cycle and apoptosis analysis. Soas-2 cells were transfected with (μg DNA per 10-cm dish): 0.5 pC53-SN-3, 1.0 pCMV12S or Δ2–36, 1.0 pCMVCD20. Total DNA added per dish was 18.5 μg. Cells were immunostained for cell-surface CD20 and propidium iodide-stained for DNA[29]. For FACS analysis, DNA content of $CD20^+$ cells was evaluated. Cells with sub-G1 content were designated the apoptotic population.

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Structure of isopenicillin *N* synthase complexed with substrate and the mechanism of penicillin formation

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The biosynthesis of penicillin and cephalosporin antibiotics in microorganisms requires the formation of the bicyclic nucleus of penicillin[1]. Isopenicillin *N* synthase (IPNS), a non-haem iron-dependent oxidase, catalyses the reaction of a tripeptide, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV), and dioxygen to form isopenicillin *N* and two water molecules[2]. Mechanistic studies suggest the reaction is initiated by ligation of the substrate thiolate to the iron centre, and proceeds through an enzyme-bound monocyclic intermediate[3,4] (Fig. 1). Here we report the crystal structure of IPNS complexed to ferrous iron and ACV, determined to 1.3 Å resolution. Based on the structure, we propose a mechanism for penicillin formation that involves ligation of ACV to the iron centre, creating a vacant iron coordination site into which dioxygen can bind. Subsequently, iron-dioxygen and iron-oxo species remove the requisite hydrogens from ACV without the direct assistance of protein residues (Fig. 2). The crystal structure of the complex with the dioxygen analogue, NO and ACV bound to the active-site iron supports this hypothesis.

Spectroscopic studies of IPNS in the resting state have suggested the presence of two or three histidines, an aspartate and possibly two water molecules as metal ligands[5,6]. The crystal structure[7] of *Aspergillus nidulans* IPNS complexed with divalent manganese (substituting for iron) revealed a metal ion octahedrally coordinated by His 214, Asp 216, His 270, Gln 330 and two water molecules (Fig. 3a). Under aerobic conditions, crystals of IPNS bound with both iron and ACV could not be obtained owing to instability and turnover problems. Crystals of IPNS complexed to ferrous iron and ACV were therefore grown under anaerobic conditions[8].

The Fe(II) : ACV : IPNS structure has one protein molecule with ferrous ion and ACV bound at the active site in the asymmetric unit (Fig. 3b). Substrate binding does not distort the 'jelly-roll' core of the enzyme, in which the iron and ACV are enclosed. The side chain of Gln 330, which coordinates the metal in the absence of substrate, is replaced by the ACV thiolate. In the Fe(II) : ACV : IPNS complex, the seven carboxy-terminal residues adopt a conformation that extends the final helix (α-10) relative to the Mn : IPNS structure and encloses the substrate in the active site.

The ACV is anchored within the active site by ligation of its thiolate to the iron centre and through its two carboxylate groups (Fig. 4a). One of the two water molecules ligating the metal ion in the Mn : IPNS complex is displaced, changing the metal coordination geometry from octahedral to square pyramidal (Fig. 2: **4** → **5**). In the substrate complex, three of the five coordination sites are filled with protein ligands: His 214, His 270 and Asp 216 (ref. 9). The remaining two sites are occupied by a water molecule (at position 398) and the ACV thiolate. The valine isopropyl group is held in van der Waals contact with the iron by interactions with Leu 231, Val 272, Pro 283 and Leu 223. The presence of the substrate-derived valine methyl group in the coordination site *trans* to Asp 216 prevents a water molecule from binding. Remarkably, the valine βC–H bond, which is cleaved during the formation of the thiazolidine ring, is directed away from the iron centre. The pentacoordiante and high-spin nature of the Fe(II) : ACV : IPNS complex, and the displacement of one water molecule on ACV binding, were previously inferred from spectroscopic studies[5,6,10].

The aminoadipoyl residue of ACV lies in an extended conformation, as predicted from analogue studies[1,3,11], and its carboxylate makes a salt bridge with Arg 87, replacing that formed between the C-terminal carboxylate (Thr 331) and Arg 87 in the Mn : IPNS structure[12].

The carboxylate of the valine residue of ACV is prevented from coordinating to the iron centre by a hydrogen-bonding network. Before ACV binds, the side chain of Arg 279 points out of the active site towards the exterior of the enzyme (Fig. 3a), but in the Fe(II) : ACV : IPNS structure (Figs 3b and 4a), Arg 279 is directed into the active-site cavity by forming a hydrogen bond to the valine carboxylate of ACV through a bridging water molecule (labelled as

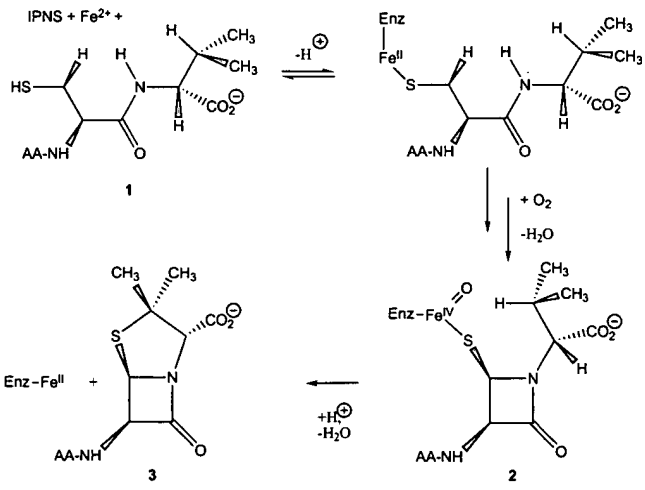

Figure 1 The IPNS reaction pathway through the proposed enzyme (enz)-bound monocyclic intermediate (**2**). AA, *L*-δ-(α-aminoadipoyl).